Biochem. J. (1992) 284, 425–431 (Printed in Great Britain)

Histamine H_1-receptor-mediated calcium influx in DDT_1MF-2 cells

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Undifferentiated monolayers of the hamster vas deferens smooth-muscle cell line, DDT_1MF-2, were grown on glass coverslips and loaded with the Ca^{2+}-sensitive fluorescent dye fura-2. Stimulation with histamine produced a rapid and maintained increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$), with an EC_{50} of $7.0 \pm 0.7\ \mu M$. The initial rise in $[Ca^{2+}]_i$ can be attributed to Ca^{2+} release from intracellular stores, whereas the maintained or plateau phase is due to influx of extracellular Ca^{2+}. The Ca^{2+} influx associated with the plateau phase required the continued presence of histamine on the receptor, since the H_1-antagonist mepyramine ($10\ \mu M$) attenuated the rise in $[Ca^{2+}]_i$ observed when extracellular Ca^{2+} was re-applied after the cells had been stimulated with histamine, in experiments performed in nominally Ca^{2+}-free buffer. Pretreatment with the inorganic Ca^{2+}-channel blockers Ni^{2+} (1 mM) and Co^{2+} (1 mM) inhibited the influx component, whereas the organic voltage-operated Ca^{2+}-channel antagonists nifedipine ($10\ \mu M$) and PN-200-110 ($10\ \mu M$) had no effect. These data suggest that histamine stimulates Ca^{2+} influx through an H_1-receptor-activated Ca^{2+} channel. Experiments with Mn^{2+} indicated that the receptor-mediated Ca^{2+}-influx pathway(s) is impermeable to Mn^{2+}. Furthermore, the refilling of Ca^{2+} stores can occur independently of H_1-receptor-mediated influx, since store refilling can be demonstrated even when the receptor-mediated Ca^{2+} entry is blocked by mepyramine. In conclusion, H_1-receptor activation in the smooth-muscle cell line DDT_1MF-2 stimulates both release of Ca^{2+} from intracellular stores [inositol 1,4,5-trisphosphate ($Ins P_3$)-mediated] and Ca^{2+} influx through a receptor-activated Ca^{2+} channel. The subsequent refilling of the $Ins P_3$-sensitive intracellular Ca^{2+} store is independent of histamine H_1-receptor stimulation (mepyramine-insensitive) and occurs without an observable rise in cytosolic free Ca^{2+}.

INTRODUCTION

Histamine H_1-receptor stimulation in a wide variety of tissue and cell types leads to an increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) [1]. In mammalian cells, H_1-receptors are generally coupled to phospholipase C, via a regulatory G-protein, which upon activation hydrolyses the plasma-membrane phospholipid phosphatidylinositol 4,5-biphosphate to inositol 1,4,5-trisphosphate ($Ins P_3$) and diacylglycerol (for reviews see [2,3]). Diacylglycerol can then activate protein kinase C, whereas $Ins P_3$ triggers the release of Ca^{2+} from intracellular stores, producing a rise in $[Ca^{2+}]_i$. In addition to $Ins P_3$-mediated intracellular Ca^{2+} release, H_1-receptor stimulation also causes a substantial influx of extracellular Ca^{2+} [1]. For example, H_1-receptor stimulation in human airway smooth muscle [4], rat vascular smooth muscle [5,6] and human umbilical-vein endothelial cells [7,8] produces a sustained rise in $[Ca^{2+}]_i$ that is biphasic in nature. The first component results from the release of Ca^{2+} from intracellular stores, whereas, the sustained or plateau phase is predominantly Ca^{2+} influx. In contrast, H_1-receptor stimulation in human fibroblasts [9], 1321N1 astrocytoma cells [10] and N1E-115 neuroblastoma cells [11,12] results in a transient rise in $[Ca^{2+}]_i$ ($Ins P_3$-mediated), with a considerably decreased or absent plateau phase.

The mechanism(s) involved in generating the Ca^{2+}-influx component of the histamine H_1-receptor response in the majority of tissue and cell types remains to be established. However, there are a variety of postulated mechanisms by which agonists can activate the entry of Ca^{2+} across the plasma membrane (for reviews see [13,14]). These include: (1) via a receptor-operated Ca^{2+} channel (i.e. receptor directly linked to a Ca^{2+} channel), an example of which is the ATP-activated Ca^{2+} channel found in rabbit arterial smooth muscle [15], (2) a voltage-operated Ca^{2+} channel, (3) a secondary messenger-activated Ca^{2+} channel (perhaps involving inositol 1,3,4,5-tetrakisphosphate [16]), (4) a G-protein-coupled Ca^{2+} channel, or (5) the Ca^{2+} entry may be 'driven' by the amount of Ca^{2+} in the intracellular calcium pool [17].

We have previously measured $[Ca^{2+}]_i$ changes in response to H_1-receptor stimulation in suspensions of DDT_1MF-2 cells [18]. The response comprised two distinct phases: (i) release of Ca^{2+} from intracellular stores ($Ins P_3$-induced) and (ii) influx of extracellular Ca^{2+} through the plasma membrane.

In the present paper we have extended our studies on histamine H_1-receptor-induced changes in $[Ca^{2+}]_i$ to undifferentiated monolayers of DDT_1MF-2 cells grown on glass coverslips, and now report that Ca^{2+} entry after H_1-receptor activation can be achieved via two separate mechanisms.

MATERIALS AND METHODS

Materials

The hamster vas deferens smooth muscle cell (DDT_1MF-2) was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, U.K.). Fura-2/AM and ionomycin were from Calbiochem. Histamine, mepyramine, bradykinin and nifedipine were obtained from Sigma, and PN-200-110 (Isradipine) and SK&F 96365 were kindly given by Sandoz (Basel, Switzerland) and SmithKline Beecham Pharmaceuticals (Welwyn, Herts., U.K.) respectively. Dulbecco's modified Eagle's medium and foetal-calf serum were from Northumbria Biologicals (U.K.). All other chemicals were of analytical grade.

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concn.; $Ins P_3$, inositol 1,4,5-trisphosphate.

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Cell culture

DDT_1MF-2 cells were cultured at 37 °C in a humidified air/CO_2 (9:1) atmosphere in 75 cm^2 flasks (Costar). The growth medium was Dulbecco's modified Eagle's medium supplemented with 2 mM-L-glutamine and 10% (v/v) foetal-calf serum. Cells were passaged twice a week (1/5 split ratio) by vigorous shaking of the flask and placed into 75 cm^2 flasks and fed with fresh growth medium every 48 h. Cells for $[Ca^{2+}]_i$ determinations were grown on 24 mm × 10 mm glass coverslips in 90 mm-diam. Petri dishes. All experiments were performed on confluent monolayers (passages 4–12, numbers assigned after receiving the cell line).

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by loading confluent cell monolayers with the Ca^{2+}-sensitive fluorescent dye fura-2. Individual coverslips were placed in 35 mm Petri dishes with 1 ml of physiological buffer (145 mM-NaCl, 10 mM-glucose, 5 mM-KCl, 1 mM-$MgSO_4$, 10 mM-Hepes, 2 mM-$CaCl_2$, pH 7.45) containing 10% foetal-calf serum and 3 μM-fura-2/AM and incubated for 30 min at 37 °C. After this 'loading' period, the fura-2-containing buffer was replaced with fresh buffer that was free of fura-2 and FCS but contained 0.1% BSA, and left at 37 °C for a further 15 mins. Loaded coverslips were then mounted in a specially designed holder which enabled the coverslip to be positioned across the diagonal of a polymethacrylate cuvette. Each cuvette contained 2.9 ml of physiological buffer (drugs were added to the cuvettes in 100 μl portions) and fluorescence measurements were made at 37 °C with a Perkin–Elmer LS 50 spectrometer. The excitation wavelengths were 340 and 380 nm, with emission at 500 nm. The slit-widths were set at 10 nm for both the excitation and emission wavelengths, and the time taken to switch between 340 and 380 nm was 0.8 s. Intracellular Ca^{2+} was calculated every 1.6 s from the ratio (R) of 340 nm/380 nm fluorescence values by using the equation of Grynkiewicz *et al.* [19]:

$$[Ca^{2+}]_i = \frac{(R - R_{min.})}{(R_{max.} - R)} \times (S_{380,min.}/S_{380,max.}) \times K_d$$

where K_d is the affinity of fura-2 for Ca^{2+} (224 nM at 37 °C) and $S_{380,min}/S_{380,max.}$ is the ratio (β value) of the fluorescence values obtained at 380 nm in the absence and presence of saturating $[Ca^{2+}]_i$. The maximum and minimum R values ($R_{max.}$ and $R_{min.}$) were determined on separate coverslips under saturating $[Ca^{2+}]_i$ (achieved by increasing the extracellular $[Ca^{2+}]$ to 20 mM, followed by 10 μM-ionomycin, pH 7.45) and Ca^{2+}-free (achieved by using 8.3 mM-EGTA, immediately followed by 25 μl of 1.0 M-NaOH to compensate for the decrease in pH, in the presence of 10 μM-ionomycin) conditions respectively. Corrections for autofluorescence were made by measuring the fluorescence produced by coverslips that had not been loaded with fura-2. Where Ca^{2+}-free conditions were required, experiments were performed in nominally Ca^{2+}-free buffer containing 0.1 mM-EGTA.

Data analysis

Agonist and antagonist concentration–response curves were fitted to a logistic equation by using the non-linear regression program GraphPAD (ISI).

RESULTS

Effects of histamine H_1-receptor stimulation on $[Ca^{2+}]_i$ in DDT_1MF-2 cells

Histamine H_1-receptor stimulation in the smooth-muscle cell line DDT_1MF-2 causes a rapid and dose-dependent increase in $[Ca^{2+}]_i$. The addition of 100 μM-histamine to monolayers of confluent undifferentiated DDT_1MF-2 cells grown on glass coverslips elicited a rapid increase in basal $[Ca^{2+}]_i$ from 100 ± 7.7 nM ($n = 15$) to approx. 650 nM, within 20 s of application (see Fig. 1*a*). The response was fairly well maintained and declined slowly towards basal levels in the presence of extracellular Ca^{2+}, i.e. 2 min after drug addition the $[Ca^{2+}]_i$ is still 350 nM (see Fig. 1*a*). Histamine concentration–response curves for the initial peak (i.e. maximum response) and later phases of the response (determined at 30 s, 60 s and 90 s after the initial peak) are shown in Fig. 2, with their accompanying EC_{50} values cited in the Figure legend. The EC_{50} values obtained for the initial peak and later phases of the response show that both components are similarly sensitive to histamine. These data agree with similar $[Ca^{2+}]_i$ experiments performed on cell suspensions of DDT_1MF-2 cells [18].

The maintenance of the response to histamine appeared to be dependent on the presence of extracellular Ca^{2+}, since experiments performed in nominally Ca^{2+}-free buffer containing 0.1 mM-EGTA resulted in a more transient response to histamine. Fig. 1(*b*) shows a profile obtained by stimulating H_1-receptors with 100 μM-histamine in the absence of extracellular Ca^{2+}; clearly $[Ca^{2+}]_i$ approaches the basal level within 2 min after stimulation. In addition, removal of extracellular Ca^{2+} causes an attenuation of the maximum response (Fig. 1*a*, compared with Fig. 1*b*). In the absence of extracellular Ca^{2+}, the response to 100 μM-HA is 69.8 ± 4.8% ($n = 6$) of that obtained in the presence of extracellular Ca^{2+}. However, if Ca^{2+} is re-applied, after the cells have been stimulated with 100 μM-histamine in the absence of extracellular Ca^{2+}, there is a rise in $[Ca^{2+}]_i$ indicative of

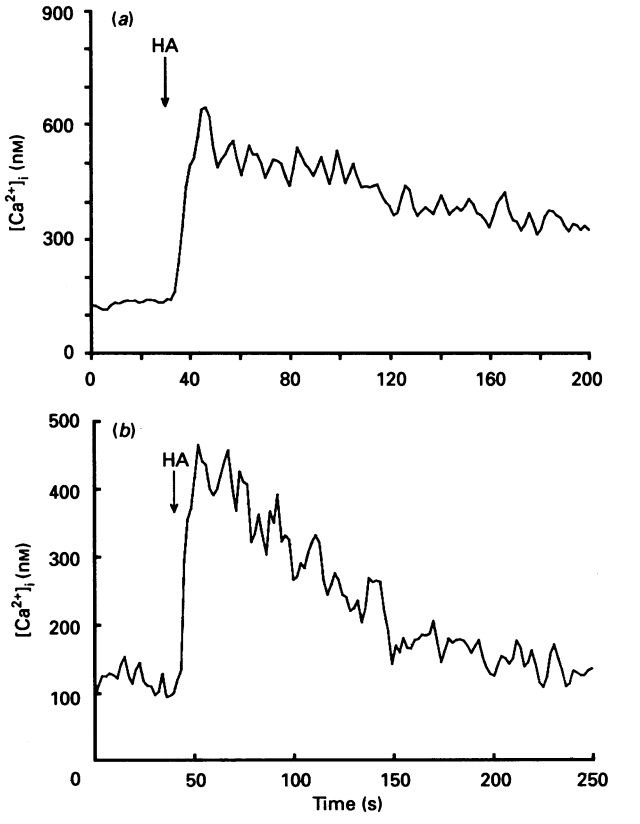

Fig. 1. Effect of H_1-receptor stimulation on $[Ca^{2+}]_i$ in fura-2-loaded DDT_1MF-2 cells

(*a*) In the presence of extracellular Ca^{2+} (2 mM); (*b*) in the presence of nominally Ca^{2+}-free buffer and 0.1 mM-EGTA. Histamine (HA; 100 μM) was added where indicated. Similar results were obtained in at least three other experiments.

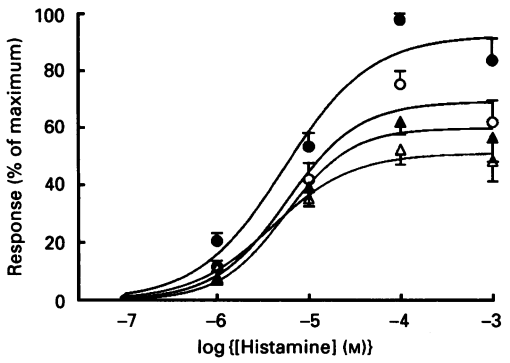

Fig. 2. Concentration–response curves for histamine-stimulated increases in $[Ca^{2+}]_i$ in DDT$_1$MF-2 cells

Profiles and accompanying EC_{50} values (μM) represent the peak ratio (F_{340}/F_{380}) response (●, 7.0±0.7) and later phases at times 30 s (○, 8.6±2.9), 60 s (▲, 6.6±1.1), and 90 s (△, 4.7±1.2) after the maximum response. The data are expressed as a percentage of the maximum response (expressed as an increase in F_{340}/F_{380} ratio minus the basal fluorescence ratio). Data are means±S.E.M. of five experiments.

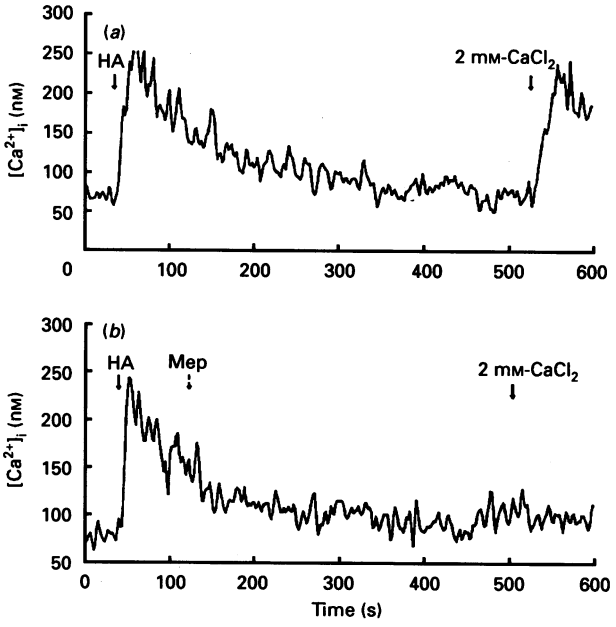

Fig. 3. Effects of re-applying extracellular Ca^{2+} (2 mM) in the absence (*a*) and presence (*b*) of mepyramine, during experiments performed in nominally Ca^{2+}-free buffer and 0.1 mM-EGTA

Histamine (HA; 100 μM), mepyramine (Mep; 10 μM) or $CaCl_2$ (2 mM) was added where indicated. Similar results were obtained in at least three other experiments.

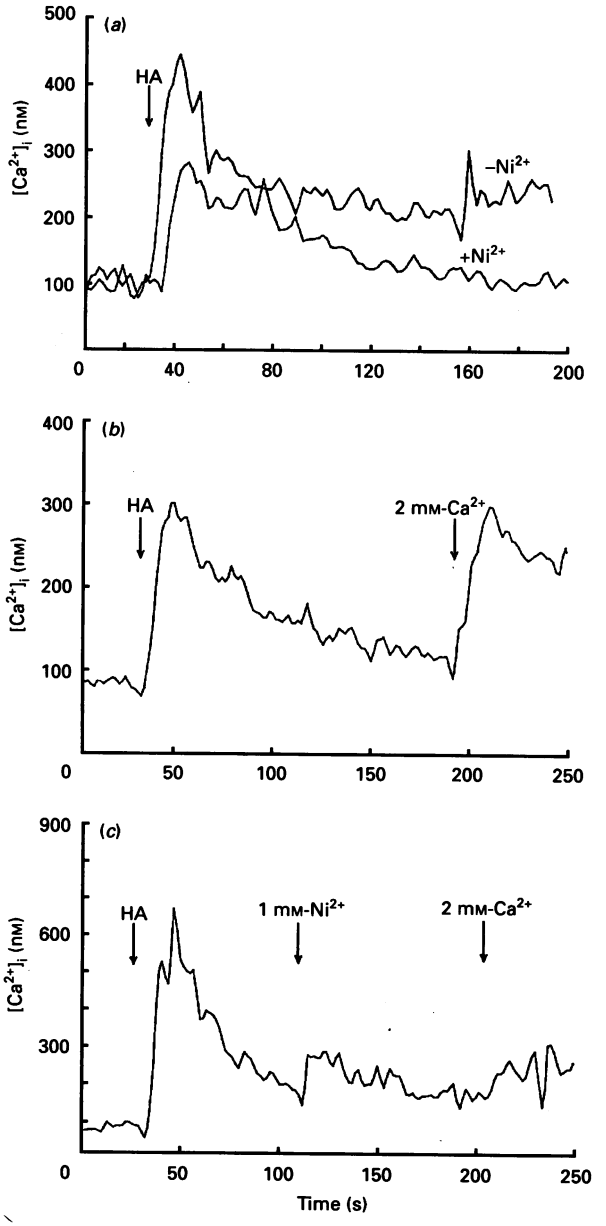

Fig. 4. Effect of Ni^{2+} on histamine-stimulated $[Ca^{2+}]_i$ changes

(*a*) Stimulation with histamine in the presence of extracellular Ca^{2+} (2 mM) after the coverslip had been preincubated for 15 min with 1 mM-Ni^{2+}. The control experiment shown for (*a*) and represented as $-Ni^{2+}$ was obtained on the same experimental day. (*b*) Control for (*c*) obtained on the same experimental day. Ca^{2+} was re-applied after stimulation with histamine in Ca^{2+}-free medium (0.1 mM-EGTA). (*c*) Effect of adding 1 mM-Ni^{2+} before re-addition of extracellular Ca^{2+} (2 mM) after the cells had been stimulated with histamine in Ca^{2+}-free buffer (0.1 mM-EGTA). Histamine (HA; 100 μM), Ni^{2+} (1 mM) or $CaCl_2$ (2 mM) was added where indicated. Similar results were obtained in three other experiments.

calcium influx (see Fig. 3*a*). To dismiss the possibility that this rise in $[Ca^{2+}]_i$ is simply a consequence of fura-2 leakage into the extracellular medium, an experiment was performed (results not shown) in which histamine was replaced with water. There was no observable increase in $[Ca^{2+}]_i$ during this experiment, indicating that the rise in $[Ca^{2+}]_i$ (after re-applying 2 mM-$CaCl_2$) shown in Fig. 3(*a*) is a result of calcium entry into the cell.

Receptor-mediated Ca^{2+} entry (influx)

We performed experiments using the H_1-antagonist mepyramine, in order to determine whether the Ca^{2+} entry (influx) that occurs during the later phase of the response to histamine is dependent on the continued presence of histamine at the H_1-receptor. Fig. 3(*b*) shows that removing histamine from the receptor (with 10 μM-mepyramine; applied 6.3 min before 2 mM-$CaCl_2$) attenuates the rise in $[Ca^{2+}]_i$ observed when Ca^{2+} (2 mM) is re-applied after the cells have been stimulated with histamine (100 μM) in the absence of extracellular Ca^{2+} (compare Fig. 3*a* with Fig. 3*b*).

Our previous data obtained with suspensions of DDT$_1$MF-2 cells [18] showed that the histamine-stimulated increase in $[Ca^{2+}]_i$

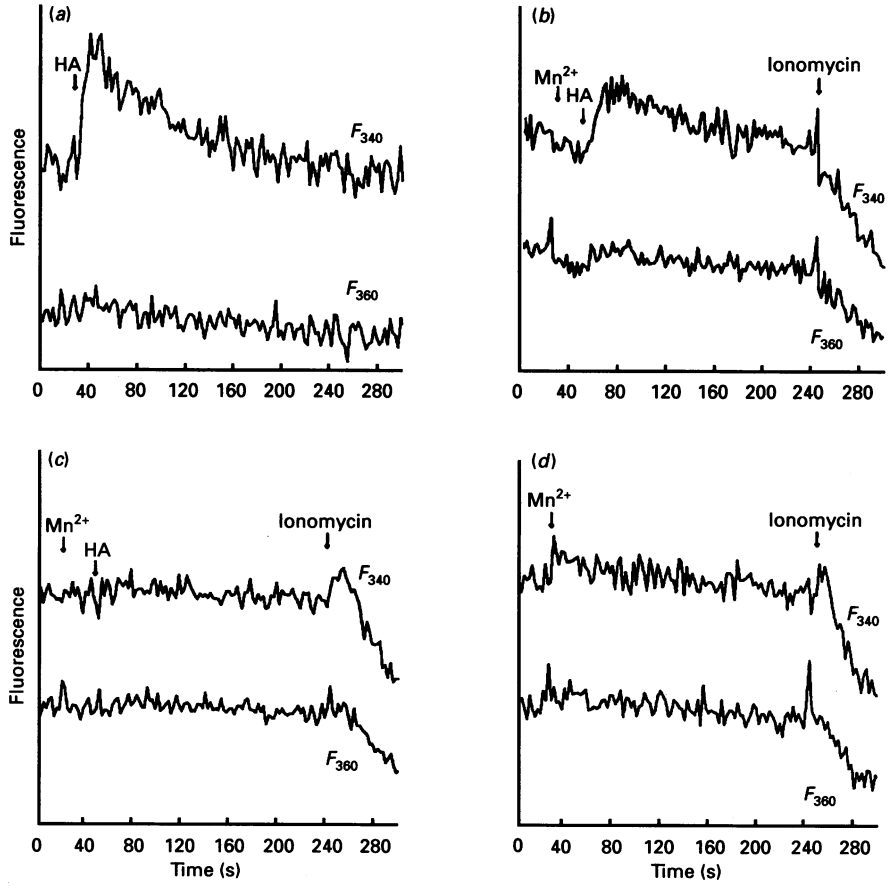

Fig. 5. Effect of Mn^{2+} in DDT$_1$MF-2 cells

(*a*) Control experiment performed in nominally Ca^{2+}-free buffer showing the fluorescence data measured at 340 nm and 360 nm when the cells were stimulated with histamine. (*b*) Effect of adding Mn^{2+} (0.1 mM) before stimulation with 100 μM-histamine in nominally Ca^{2+}-free buffer. (*c*) Effect of adding Mn^{2+} (0.1 mM) before 100 μM-histamine in nominally Ca^{2+}-free buffer to cells that had been preincubated for 15 min with 100 nM-mepyramine. (*d*) Effect of adding Mn^{2+} (0.1 mM) alone in nominally Ca^{2+}-free buffer. Addition of ionomycin at the end of (*b*), (*c*) and (*d*) resulted in a rapid quenching of the fluorescence at 340 nm and 360 nm. Histamine (HA; 100 μM), Mn^{2+} (0.1 mM) and ionomycin (10 μM) were added where indicated. Similar results were obtained in three other experiments.

involves the 'classical' histamine H_1-receptor (i.e. K_d values of the order of 1 nM [20]). Similar results (not shown) were obtained in the present study, the peak and later phase (measured 90 s after the peak) of the response being equally sensitive to mepyramine, with IC_{50} values of 5.4 ± 0.6 nM ($n = 3$) and 3.00 ± 0.8 nM ($n = 3$).

Effects of organic and inorganic Ca^{2+}-channel antagonists on the receptor-mediated Ca^{2+} influx

The characteristics of the Ca^{2+} channel involved in the receptor-mediated Ca^{2+} entry were examined in a series of experiments using organic and inorganic Ca^{2+}-channel blockers. Preincubation with the organic dihydropyridine voltage-operated Ca^{2+} channel antagonists nifedipine (10 μM) or PN-200-110 (10 μM) had no effect on the sustained Ca^{2+} influx (results not shown), whereas pretreatment with the inorganic Ca^{2+}-channel blockers Ni^{2+} (1 mM) and Co^{2+} (1 mM) decreased the receptor-mediated Ca^{2+} influx (see Fig. 4*a*). A further consequence of preincubation with Ni^{2+} and Co^{2+} is the attenuation of the maximum signal, i.e. the response to 100 μM-histamine was $59.7 \pm 9.3\%$ ($n = 3$) and $59 \pm 9.8\%$ ($n = 3$) of that obtained in the absence of Ni^{2+} and Co^{2+} respectively. Furthermore, the addition of Ni^{2+} blocked the calcium influx observed when extracellular Ca^{2+} was re-applied, after the cells had been stimulated with 100 μM-histamine in nominally Ca^{2+}-free buffer (see Fig. 4*c*). Finally, preincubation with the proposed receptor-operated Ca^{2+}-channel blocker SK&F 96365 [21] (10 μM; $n = 3$, results not shown) had no effect on the sustained Ca^{2+} influx.

Histamine does not stimulate Mn^{2+} entry in DDT$_1$MF-2 cells

The bivalent cation Mn^{2+} is widely used as an indicator of Ca^{2+} entry into many cell types, since Mn^{2+} quenches fura-2 fluorescence at all wavelengths. Fig. 5(*a*) shows the raw fluorescence-intensity traces at 340 nm and 360 nm excitation obtained in nominally Ca^{2+}-free buffer. The addition of 100 μM-histamine causes a rapid increase at the Ca^{2+}-sensitive 340 nm wavelength, whereas the fluorescence intensity at the Ca^{2+}-insensitive 360 nm wavelength remains unchanged. The addition of Mn^{2+} (0.1 mM) at the onset of a similar experiment (see Fig. 5*b*) had a negligible effect on the fluorescence at both wavelengths; however, subsequent stimulation with 100 μM-histamine increased the fluorescence intensity at 340 nm, but had no effect at the Ca^{2+}-insensitive 360 nm wavelength. This is in contrast with ionomycin (10 μM), which rapidly caused a decrease in fluorescence intensity at both wavelengths (see Fig. 5*b*). The effect of adding Mn^{2+} (0.1 mM) after the addition of the H_1-receptor antagonist mepyramine (100 nM) is shown in Fig. 5(*c*). As would be expected, the addition of histamine in the presence of 100 nM-mepyramine (15 min preincubation) did not increase the fluorescence intensity at 340 nm, and the subsequent addition of Mn^{2+} had no effect on the 340 nm or 360 nm fluorescence intensity; however, as in Fig. 5(*b*) ionomycin (10 μM) rapidly decreased the fluorescence in-

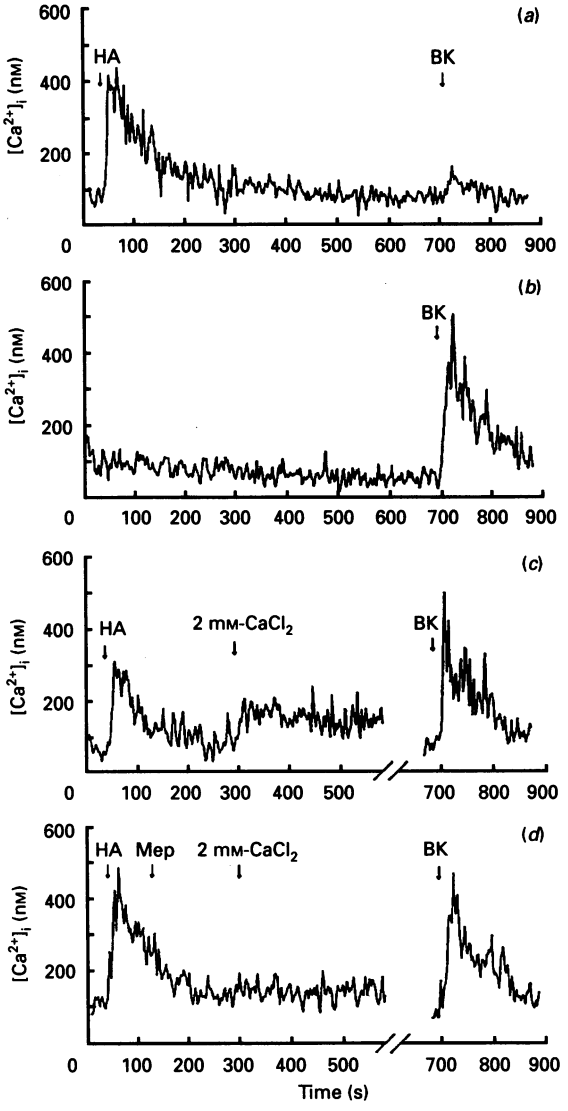

Fig. 6. Refilling of intracellular Ca^{2+} stores

Except where indicated, experiments (*a*)–(*d*) were performed in nominally Ca^{2+}-free buffer containing 0.1 mM-EGTA. The bradykinin and histamine responses were obtained in Ca^{2+}-free medium (0.1 mM-EGTA). (*a*) Stimulation with 100 μM-histamine depletes the intracellular Ca^{2+} store, since subsequent addition of bradykinin causes a negligible response. (*b*) Control experiment for (*a*) showing that bradykinin is able to elicit a normal response in the absence of the histamine response. (*c*) Re-addition of exogenous Ca^{2+} (2 mM) after histamine stimulation results in the re-appearance of the plateau phase. The subsequent addition of bradykinin now produces a normal response; the exposure to extracellular Ca^{2+} for 5 min was sufficient to refill the internal Ca^{2+} stores. (*d*) The same protocol as in (*c*), but mepyramine was added 3 min before re-addition of exogenous Ca^{2+}. The plateau phase is abolished, but the subsequent addition of bradykinin again produces a normal response. The stores have refilled independently of the receptor-mediated Ca^{2+} entry. The break in the profile observed in (*c*) and (*d*) is a consequence of replacing the cuvette medium (2 mM-Ca^{2+}) by nominally Ca^{2+}-free buffer, before stimulation with bradykinin. Histamine (HA; 100 μM), bradykinin (BK; 100 nM), $CaCl_2$ or mepyramine (Mep; 10 μM) were added where indicated. Similar results were obtained in five other experiments.

tensity of both wavelengths. Finally, the effect of adding Mn^{2+} (0.1 mM) alone is shown in Fig. 5(*d*). This experiment was routinely performed to control for any small and gradual declines in the fluorescence intensity (at both wavelengths) which were occasionally observed in some cell preparations after addition of Mn^{2+} (owing to either a slow influx of Mn^{2+} into the cells or leakage of some fura-2 into the extracellular buffer). These experiments indicate that, although histamine stimulates Ca^{2+} entry in DDT_1MF-2 cells, the influx pathway is not permeable to Mn^{2+}.

Refilling of intracellular Ca^{2+} stores is independent of receptor-mediated Ca^{2+} influx

The data presented thus far indicate that in DDT_1MF-2 cells histamine stimulates Ca^{2+} influx into the cytoplasm through a receptor-mediated Ca^{2+} channel. This raised the question as to whether the receptor-mediated Ca^{2+} influx is used to refill the InsP_3-depleted intracellular Ca^{2+} store or whether refilling of the intracellular Ca^{2+} store can occur independently of the receptor-mediated Ca^{2+} influx. In order to investigate this, DDT_1MF-2 cells were initially stimulated with 100 μM-histamine in nominally Ca^{2+}-free buffer, after which exogenous Ca^{2+} was re-applied in the absence or presence of the H_1-antagonist mepyramine. Finally, the cells were challenged, in nominally Ca^{2+}-free buffer, with a second Ca^{2+}-mobilizing (InsP_3-mediated) agonist (i.e. bradykinin).

Fig. 6(*a*) shows the effect of adding bradykinin (100 nM) after the cells had been stimulated with 100 μM-histamine in nominally Ca^{2+}-free buffer. The bradykinin response is negligible compared with the control (Fig. 6*b*), suggesting that exposure to 100 μM-histamine was sufficient to deplete the intracellular Ca^{2+} stores that would normally be available for the InsP_3 generated by activation of the bradykinin B_2-receptor.

Fig. 6(*c*) shows the effect of a similar protocol to that in Fig. 6(*a*), but with the inclusion of a 5 min period between the stimulations by histamine and by bradykinin where 2 mM-extracellular Ca^{2+} was added. As shown in Fig. 3(*a*), the re-addition of extracellular Ca^{2+} results in re-appearance of the plateau phase. If the cells are now exposed to bradykinin (100 nM), in the absence of extracellular Ca^{2+} (the glass coverslip was transferred to a fresh cuvette that contained nominally Ca^{2+}-free buffer), there is a substantial increase in $[Ca^{2+}]_i$ which is attributed to release of Ca^{2+} from intracellular stores (InsP_3-mediated). The 5 min exposure to extracellular Ca^{2+} before addition of bradykinin appears to have been sufficient to refill the intracellular Ca^{2+} store.

Finally, to determine whether the refilling of intracellular Ca^{2+} stores can occur independently of any receptor-mediated Ca^{2+} entry, the H_1-antagonist mepyramine was added before re-addition of exogenous Ca^{2+}. Fig. 6(*d*) shows the effect of exposing the monolayer to mepyramine (10 μM) 3 min before the re-addition of extracellular Ca^{2+} (2 mM), after the cells had been stimulated with 100 μM-histamine in the absence of extracellular Ca^{2+}. As previously shown in Fig. 3(*b*) mepyramine attenuates the observed rise in $[Ca^{2+}]_i$ seen when Ca^{2+} (2 mM) is re-applied (Fig. 3*a* compared with Fig. 3*b*, and Fig. 6*c* compared with Fig. 6*d*). However, when the cells are subsequently stimulated with bradykinin (100 nM) in the absence of extracellular Ca^{2+} (coverslip again transferred to a fresh cuvette that contained nominally Ca^{2+}-free buffer), the response is similar to that observed in Fig. 6(*c*), where the receptor-mediated Ca^{2+} entry was not blocked by the addition of mepyramine. Therefore the intracellular Ca^{2+} stores have refilled in the presence of the H_1-antagonist mepyramine by a pathway that does not appear to involve a measurable increase in $[Ca^{2+}]_i$. These experiments suggest that in DDT_1MF-2 cells (*a*) refilling of the InsP_3-sensitive intracellular Ca^{2+} store can occur in the absence of the receptor-mediated Ca^{2+} influx pathway, and (*b*) the refilling of intracellular Ca^{2+} stores occurs via a mechanism does not involve $[Ca^{2+}]_i$ rising measurably above the basal level of 100 nM.

DISCUSSION

The data presented in this study clearly show that histamine H_1-receptor activation in undifferentiated monolayers of the vas deferens smooth-muscle cell line DDT_1MF-2 stimulates a rapid increase in $[Ca^{2+}]_i$, similar to that previously reported with suspensions of DDT_1MF-2 cells [18,22]. This histamine-stimulated increase in $[Ca^{2+}]_i$ comprises two distinct components: (1) release of Ca^{2+} from intracellular stores which is mediated by the secondary messenger $InsP_3$ (T. E. White & S. J. Hill, unpublished work), and (2) influx of extracellular Ca^{2+} through Ca^{2+} channels in the plasma membrane. Since the influx of extracellular Ca^{2+} associated with the sustained phase of the histamine response can be inhibited by the H_1-antagonist mepyramine (see Fig. 3*b*), the Ca^{2+} channel involved will be referred to as a receptor-activated Ca^{2+} channel.

In DDT_1MF-2 cells, the receptor-activated Ca^{2+} influx could be blocked by the inorganic ions Co^{2+} and Ni^{2+} (see Fig. 4*a*), but not by the dihydropyridine voltage-operated Ca^{2+}-channel antagonists nifedipine and PN-220-110. These data are similar to those recently reported in human airway smooth muscle [4], although the relationship between the receptor-activated Ca^{2+} influx and the refilling of intracellular Ca^{2+} stores remains to be established. A further characteristic of the receptor-mediated Ca^{2+} entry in DDT_1MF-2 cells and human airway smooth muscle is that there is no observable Mn^{2+} influx associated with the histamine response. Interestingly, Mn^{2+} entry was not detected in parotid cells stimulated with carbachol, even though receptor-mediated Ca^{2+} entry was strongly indicated by other data [23]. These data contrast with those obtained in human umbilical-vein endothelial cells where histamine H_1-receptor activation stimulates the entry of Mn^{2+} into the cytoplasm [7]. However, inspection of the data presented by Hallam *et al.* [8] shows that H_1-receptor activation in human umbilical-vein endothelial cells stimulates both Ca^{2+} entry into the cytoplasm which can be inhibited by mepyramine (receptor-activated Ca^{2+} influx) and Ca^{2+} entry in the cytoplasm which is independent of receptor stimulation [8], i.e. a transient elevation in $[Ca^{2+}]_i$ was observed during an experiment performed by a protocol similar to that in Fig. 6(*d*). In addition, Hallam *et al.* [8] proposed that the refilling of intracellular stores is controlled not by the stimulation of cell-surface receptors but by the state of fullness (or depletion) of intracellular stores of Ca^{2+}. Interestingly, the Mn^{2+}-influx pathway in human umbilical-vein endothelial cells could be activated independently of continued H_1-receptor stimulation (i.e. during refilling of the intracellular Ca^{2+} store).

In DDT_1MF-2 cells histamine H_1-receptor activation can also lead to both (i) a Ca^{2+} influx into the cytoplasm that is dependent on continued H_1-receptor occupancy (receptor-mediated) and (ii) Ca^{2+} influx into the intracellular store that is independent of H_1-receptor occupancy. The one notable difference from the mechanisms operating in umbilical endothelial cells, however, is that the refilling of the intracellular store does not involve $[Ca^{2+}]_i$ rising measurably above the basal level of 100 nM. The refilling mechanism may involve Ca^{2+} entry directly into the intracellular store, analogous to the 'capacitative' influx mechanism described in parotid acinar cells [17]. Alternatively, Ca^{2+} ions entering the cytoplasm may be resequestered into the intracellular store at a rate that prevents a measurable rise in $[Ca^{2+}]_i$ occurring. Some support for the proposal that the refilling of the intracellular Ca^{2+} store involves Ca^{2+} entry directly to the store is provided by the work of Bian *et al.* [24]. They reported in DDT_1MF-2 cells that GTP activates the movement of Ca^{2+} between distinct $InsP_3$-sensitive and -insensitive Ca^{2+} pools and that GTP may stimulate Ca^{2+} entry across the plasma membrane directly into an $InsP_3$-sensitive Ca^{2+} pool. Recently, however, Pandol *et al.* [25] proposed that cyclic GMP is a potential soluble messenger that communicates the Ca^{2+} state of the internal store to the plasma membrane to stimulate Ca^{2+} entry. Thus there may be no need for a physical association between the intracellular store and the plasma membrane. In this case, refilling of the intracellular Ca^{2+} stores in DDT_1MF-2 cells (in the presence of mepyramine, without a measurable rise in whole-cell cytoplasmic $[Ca^{2+}]$) might be achieved via a very localized rise in cytoplasmic $[Ca^{2+}]$ if the intracellular stores are localized very close to the plasma membrane, or (as mentioned above) by a close matching of the rates of influx into the cytoplasm and resequestration into the intracellular stores.

The mechanism(s) by which agonists activate receptor-mediated Ca^{2+} influx have not been elucidated. Recently, studies using pig aortic microsomes have revealed a receptor-mediated Ca^{2+} channel which can be activated by histamine and the stable GTP analogue guanosine 5′-[$\beta\gamma$-imido]triphosphate, but is inhibited by cyclic GMP-dependent protein kinase [26,27]. These data suggest the existence of a receptor-activated Ca^{2+} channel coupled to a G-protein. Also, when human intestinal epithelial cells were pretreated with pertussis toxin, Ca^{2+} influx in response to leukotrienes D_4 and E_4 was inhibited, whereas the $InsP_3$ generation and intracellular Ca^{2+} release were unaffected [28]. The putative receptor-mediated Ca^{2+} channel may be activated by the proposed secondary messenger inositol 1,3,4,5-tetrakisphosphate [16], since H_1-receptor activation in DDT_1MF-2 cells stimulates the production of the latter [29], or by an unknown signal that is generated when the $InsP_3$-sensitive Ca^{2+} store is depleted.

In summary, the present study has shown that histamine H_1-receptor activation in undifferentiated monolayers of DDT_1MF-2 vas deferens smooth-muscle cells stimulates Ca^{2+} release from intracellular stores and Ca^{2+} entry (influx) across the plasma membrane by two different mechanisms: (i) influx into the cytoplasm, which requires the continued presence of histamine on the receptor (receptor-mediated Ca^{2+} influx), and (ii) influx into the intracellular Ca^{2+} store, which is independent of receptor occupancy.

We thank the Wellcome Trust and Medical Research Council for financial support.

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Received 17 October 1991/9 December 1991; accepted 2 January 1992